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Effect of Technique and Darkness on the Success of Meristem Micrografting of Picea abies

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Abstract

The possibility of micrografting *in vitro* shoot meristems of *Picea abies* were investigated on a 18 year-old Norway spruce clone. The rates of success were shown to be greatly influenced by the grafting technique used and by light, with a positive effect of a 2 to 3 week darkness period applied to the stocks just after they had been grafted. Average scores of more than 50% of success were obtained. However, substantial variability in terms of shoot expansion among the grafted plants existed *in vitro*, as well as after transfer to *ex-vitro* conditions.

Key words: micrografting, *Picea abies*, shoot meristem, technique, tissue culture, vegetative propagation.

FDC: 165.442; 174.7 Picea abies.

Introduction

There is much interest in favor of micrografting as broadly reviewed by BURGER (1984) and JONARD (1986). With special regard to coniferous species, most of the work carried out in this field to date mentions the use of vegetative buds or shoot tips as scions (MISSON and GIOT-WIRGOT, 1985; TRANVAN and DAVID, 1985; EWALD et al., 1991; TRANVAN et al., 1991; HUANG et al., 1992; PULLMAN and TIMMIS, 1992), but micrografting of shoot meristems has been restricted thus far to only a limited number of species (MONTEUUIS, 1986; DUMAS et al., 1989; GOLDFARB et al., 1993), despite the obvious benefits of miniaturizing the size of the grafted scion to the meristem. Meristem micrografting combines the advantages of grafting (CHAMPAGNAT, 1980), with those of meristem culture, still problematic in practice for mature conifers (PULMANN and TIMMIS, 1992) to which it can constitute a helpful substitute. The possibility of introducing into tissue culture conditions contamination-free explants through grafted meristems derived from mature genotypes, while stimulating the potential for cloning of such introduced selected plant material at the same time (FRANCLET, 1983; TRANVAN et al., 1991; HUANG et al., 1992) must be considered a major argument for this technique. In addition, grafting meristematic tissues may help in reducing compatibility problems between the scion and the stock (LACHAUD, 1975; MOORE, 1984; JONARD, 1986).

The prospects of applying this attractive meristem micrografting technology to Norway spruce (*Picea abies* (L.) KARST.), a major forest species, were analysed and are reported in this paper.

Material and Method

Obtaining in vitro rootstocks

The *in vitro* seedlings used as rootstocks were obtained from *Picea abies* seeds that were surface-sterilized by immersion in 38 % hydrogen peroxide solution for 20 min, then rinsed 3 times in sterile distilled water before being individually inoculated into glass test tubes (25 mm x 200 mm) onto a 20 mm x 30 mm cellulosic "Sorbarod" plug (Baumgartner Papier SA, Lausanne. Switzerland). These Sorbarods had previously been saturated with 5 ml of liquid medium consisting of MARGARA (1977) macronutrients, MURASHIGE and SKOOG (1962) micronutrients diluted twice, 20 g/l sucrose and 10 g/l activated charcoal, before being autoclaved at 120 °C for 20 min.

The cultures were then maintained under a 16 h photoperiod with photon flux density for 110 μmol m^{-2} s^{-1} provided by "Sylvania Cool White" fluorescent lamps 36 W, and at 25/22 ± 2 °C, light/dark. Under these conditions, 50 % to 70 % of seeds that germinated developed within 2 to 3 months into young seedlings with fully expanded cotyledons and an elongating epicotyl that corresponded to the suitable stage to be grafted.

Scion origin

The apical meristems used as scions originated from vegetative buds produced by shoots of rooted cuttings of one AFOCEL superior clone of Norway spruce aged 18-years since seed germination. These 3-year to 4-year-old rooted cuttings were intensively maintained cultivated and hedged in large containers in a greenhouse with minimum temperature of 10 °C and permanent additional lighting provided by high-pressure sodium lamps.

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Several sample collections were carried out at different dates during the year.

Grafting technique

An soon as collected, the buds with a short portion of shoot underneath were dipped for a few seconds in a 70 % ethanol solution. The buds were then dissected aseptically and the apical meristems excised under a binocular microscope using a cold light source. The size of the apical meristems ranged from 100 μm to 250 μm in height and from 200 μm to 450 μm in width depending on growth phase of the shoot apex at the time of removal.

Two different micrografting techniques were compared (*Figure 1*): (a) the „side-grafting" originally developed on *Sequoiadendron giganteum* (Monteuuis, 1986) and consisting of inserting the excised meristem with a short wedge of underlying tissues into a 2 mm to 3 mm long vertical cut made on the elongating epicotyl of the in vitro seedling used as rootstock; (b) the "top-grafting" as initially described by Navarro et al. (1975) and consisting of placing the horizontal cut section of the excised meristem onto the top cut surface of the decapitated young epicotyl of the *in vitro* seedling rootstock. The overall size of the scion did not exceed 450 μm in width and 250 μm in height or 500 μm when removed with the basal wedge (side-grafting technique).

As soon as grafted for both procedures half of the rootstocks were placed under the same environmental conditions as formerly described, while the other half was kept for 2 to 3 weeks in darkness before being transferred to the standard conditions.

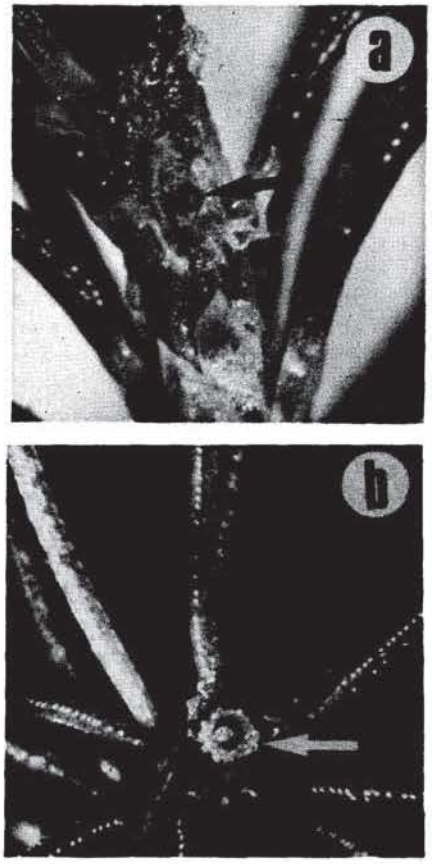

Figure 1. — Recently micrografted meristems of *Picea abies* (arrowheads) illustrating the "side-grafting" (a) and the "top-grafting" (b) techniques described in the text.

Figure 2. — Elongating scion deriving from a side-grafted meristem after the rootstock epicotyl had been cut back just above the graft point.

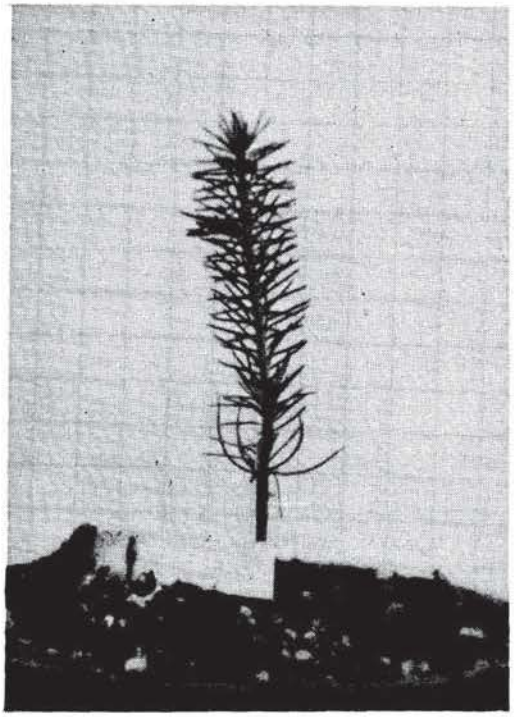

Figure 3. — Acclimated Picea abies micrograft. The sides of the background squares are 1 cm long.

The influence of darkness on grafting success was more precisely investigated by applying the same protocol to the side-grafted plants only.

Care of the grafted plants and acclimatization

Every 2 to 3 months, as required, 2 ml of the sterilized liquid medium was provided under aseptic conditions to the grafted rootstocks kept cultivated in their original test tube. In addition, soon after the side-grafted meri-

Table 1. — Micrografting success scores for side-grafting and top-grafting techniques applied to shoot meristems removed at different dates from an 18-year-old *Picea abies* genotype.

Dates of scion collection / grafting	Side-grafting	Top-grafting
13-6-1989	4/12	1/12
30-6-1989	6/12	0/10
25-7-1989	7/12	0/12
9-8-1989	8/12	2/12
29-12-1989	5/18	1/17
Average rate of success	**30/66**	**4/63**
% ± S.D.	**45.5 ± 6.1**	**6.3 ± 3.0**

SD: standard deviation

stem started new organogenesis demonstrating that connection with the stock had occurred, the stock epicotyl was cut off just above the graft point (*Figure 2*). Also any axillary shoot produced by the stock, whatever the type of grafting would be systematically removed to avoid competition.

Transfer of the grafted plants to the greenhouse was achieved preferably in spring, by carefully removing the sorbarod by hand before transplantation into a horticultural peat-perlite (50:50, v/v) substrate watered with a fungicide solution and covered with a thin plastic film to maintain sufficiently high relative humidity. This plastic cover was progressively removed after 2 weeks to 4 weeks to get the grafted plants acclimatized (*Figure 3*).

Results

The rate of success, in terms of meristems exhibiting organogenic capacity after they had been micrografted, was shown to be strongly influenced by the technique used, the side-grafting giving rise to 45.5 % of positive responses against 6.3 % for the top-grafting technique (*Table 1*, $P < 0.001$ as the result of the Chi-square test).

The beneficial influence of placing the grafted *in vitro* seedlings for 2 weeks to 3 weeks in darkness immediately after grafting resulted in average success rates of 52.4 % as compared to 32.6 % for the control in standard lighting conditions (*Table 2*, $P < 0.01$, Chi-square test).

However, it appears from *table 1* and *table 2* that the scores were susceptible to variation from one date of experiment to another.

Whatever the procedure used, the successful micrografts exhibited substantial variability in terms of further development of the scion, from a resting scaly bud to an actively expanding juvenile-like shoot. Such noticeable variability was observed even for meristems removed at the same date from the mother plant.

The grafted plants were acclimatized to *ex-vitro* conditions without any serious problem.

Discussion

The success of meristem micrografting of Norway spruce was shown to be largely dependent on the procedure used. The side-grafting technique appeared to be more efficient. It required high dexterity from the manipulator. Ability to draw the rootstock seedling out of the tube to facilitate manipulations without damaging its root system was a determining factor. This was possible thanks to the rod used as physical support. Furthermore, the better quality of the roots that developed in the "Sorbarod" made the transfer to the horticultural substrate easier. This allowed grafting onto juvenile seedlings, which can be considered more suitable than older material or unrooted microcuttings, giving higher rates of grafting success (Monteuuis and Dumas, unpublished results) and especially in view of the aim to recover juvenile potentialities from the grafted meristem assuming a transfer of graft-transmissible juvenility promoting substances from the young seedling used as stock (Champagnat, 1980; Dole and Wilkins, 1991).

In contrast to *Pinus pinaster* (Dumas et al., 1989), but in the same way as for *Sequoiadendron giganteum* (Monteuuis, 1986), the meristem had to be removed with a small wedge of underlying tissues to be inserted into the small cut previously made on the rootstock epicotyl to keep it in tight contact with the stock until the connection occured. The influence of these underlying tissues on the possibility of the grafted meristem to recover juvenile potentialities would require further analysis. But it seems logical to assume that the tinier the scion, the more damaging the excision and the more limited the endogenous resources needed until the connection with the stock has occurred thereby decreasing grafting success as demonstrated by Navarro et al. (1975) on *Citrus*, Huang and Millikan (1980)

Table 2. — Effect of 2 week post-grafting darkness on the success scores of meristem side-micrografts of an 18 year-old *Picea abies* genotype performed at different dates.

Dates of scion collection / grafting	Standard lighting conditions	Darkness
30-6-1989	2/6	4/6
25-7-1989	3/6	4/6
9-8-1989	4/6	4/6
24-8-1989	7/11	11/11
11-9-1989	7/9	4/7
27-10-1989	4/8	8/8
23-11-1989	2/11	4/10
11-12-1989	0/12	0/12
20-12-1989	0/11	0/9
29-12-1989	0/9	5/9
Average rate of success	**29/89**	**44/84**
% ± S.D.	**32.6 ± 5.0**	**52.4 ± 5.4**

SD: standard deviation

on *Malus*, and MONTEUUIS (1987) on *Sequoiadendron giganteum*. This could explain the failure of the meristems top-grafted onto the decapitated stocks, beside also the fact that the contact between the tissues of the 2 partners were not as tight as for the side-grafting method.

Endogenous growth regulators have been supposed to play a key role in grafting with special references to auxin (SHIMOMURA and FUZIHARA, 1977; MOORE, 1984) which is known to be synthetized in shoot apices and degraded by light. This could constitute an hypothesis in favor of the beneficial effect of placing the grafted plants in darkness. in the same way as TRANVAN and DAVID (1985) and TRANVAN et al. (1991), allowing for the time needed for the connection to occur. Light may also affect the excised meristem causing irreparable stress and photooxydation reactions.

The substantial intraclonal variability observed among the successful micrografted plants has been reported for other species when applying the same technology (MONTEUUIS, 1986; DUMAS et al., 1989; MONTEUUIS and DUMAS, 1992). It can be caused by several factors such as the variable quality of the graft connection, the genetic diversity of the seedlings used as rootstocks and the physiological status of the excised meristems at the time of its removal to be grafted. This latter parameter in particular seems worth requiring special attention with a view to remedying this undesirable variability. We deliberately decided to concentrate our efforts on 1 *Picea abies* clone with the purpose of minimizing this variability, at least from the standpoint o fthe scion source genotype. Another factor that obviously needs to also be considered is the influence of the *in situ* location of the scion within the donor plant.

Additional experiments have however established that this meristem micrografting technique can be successfully applied to other genotypes of Norway spruce and even likely to other related species.

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Growth Rhythm and Hardiness of Picea abies Progenies of High Altitude Parents from Seed Produced at Low Elevations

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Abstract

A complete diallel was performed by crossing Norway spruce grafts of high altitude parents at a low elevation site. The families were planted in a short term field trial. Freezing tests were performed with ten controlled cross families and comparable controls.

Measurements were made of the annual shoot elongation patterns at ages 9 and 10 years from seed in the field trial and final tree heights at age 10 years. The trees of the diallel full-sib families did not have the timing and duration of shoot elongation that is to be expected of high altitude Norway spruce trees and performed similarly to trees of low altitude provenances. In diallel analyses of variance, significant reciprocal effects were found for the shoot elongation characteristics and height growth, indicating a non-symmetric genetic contribution from the 2 parents. In the freezing tests, high altitude families from crosses at low elevation sites were significantly more damaged than control provenances from the same or lower altitudes as the origins of the parents.

The present results confirm earlier observations that progenies of high altitude parents do not retain the annual growth rhythm of their parents when the parents have been grown at a low altitude site. The results are similar to observations after transfer of clones from a northern to a southern location where crosses are made.

Key words: diallel cross, sexual reproduction, environmental preconditioning, adaptation.

FDC: 165.3; 165.4; 174.7 *Picea abies.*

Introduction

Seed orchards intended to produce seed for northern latitudes or high altitudes are often located in a more favourable climate in order to enhance seed production. After transfers of parent clones from northern to southern Norway, seedlings from seeds produced in a Norway spruce (*Picea abies* (L.) KARST.) seed orchard after controlled crosses did not have the adaptational properties of the parents (JOHNSEN, 1989a and b; JOHNSEN et al., 1989). They had in particular an extended growth season and later bud-set and were more damaged in freezing tests than related seedlings from seeds produced in the northern environment. Later similar effects have been demonstrated in the same species after identical crosses performed both in southern and northern Finland (SKRØPPA et al., 1994) and inside and outside a greenhouse (JOHNSEN et al., 1994). Effects of different crossing locations have also been demonstated in Scots pine (DORMLING and JOHNSEN, 1992; LINDGREN and WEI, 1994).

Clones from high altitudes grafted at a lowland site experience climatic changes similar to the north-south transfers (JOHNSEN, 1988). Results from experiments with families after controlled crosses under such conditions are reported in this study. The crosses and a field trial were planned for other purposes, but are here utilized to study the performance of progenies produced after altitudinal tranfers of the parents.

Materials and Methods

Experimental material

In the spring of 1970, controlled crosses were performed in a grafted clonal archive of Norway spruce at Haga, southeastern Norway, altitude 100 m. The crossing design was a complete diallel involving 5 parents and included both self-fertilized and reciprocal cross families. In addition, each parent was crossed with a mixture of pollen from 5 other clones in the archive. Pollen for the crosses was produced in the clonal archive the same year. Sufficient seed for experiments was obtained from 18 outcrossed and 4 self-fertilized families from the diallels.

The 5 parent trees in the diallel originated from altitudes ranging between 620 m and 850 m in southern Norway. The parents contributing pollen to the pollen mix originated from altitudes between 680 m and 720 m in the same area. All the parents had been grafted in the clonal archive in 1964 with scions collected from plus trees in natural stands. They had been phenotypically selected